

and Differential Utilization of E Proteins

Jian-Min Zhang,* Lihsia Chen,† Michael Krause,‡
Andrew Fire,§ and Bruce M. Paterson*,¹

*Laboratory of Biochemistry, NCI, and ‡Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892; †Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710; and §Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210

The formation of striated muscle in both vertebrates and invertebrates involves the activity of the MyoD family of basic-helix-loop-helix (bHLH) transcription factors. The high degree of evolutionary conservation of MyoD-related proteins, both in the sequence of their bHLH domains and in their general developmental expression patterns, suggests that these factors are also conserved at the level of function. We have addressed this directly using MyoD and E protein factors from vertebrates, *Drosophila*, and *Caenorhabditis elegans*. Various MyoD and E factor combinations were tested for their ability to interact *in vitro* and to function *in vivo* in the myogenic conversion of 10T $\frac{1}{2}$ mouse fibroblasts. We found that the ability of different homo- and heterodimers to bind DNA *in vitro* was an accurate measure of biological activity *in vivo*. A second assessment of conserved function comes from the ability of these factors to rescue a *C. elegans* *hlh-1* (CeMyoD) null mutation. We found that both *Drosophila* and chicken MyoD-related factors were able to rescue a *C. elegans* CeMyoD loss-of-function mutation. These results demonstrate a remarkable degree of functional conservation of these myogenic factors despite differences in E-protein interactions. © 1999 Academic Press

Key Words: MyoD; E proteins; myogenesis; *Drosophila*; *C. elegans*.

INTRODUCTION

The MyoD family of basic-helix-loop-helix (bHLH) transcription factors has been shown to play an important role in striated muscle formation in both vertebrates and invertebrates. In the vertebrates, four family members have been identified: MyoD, Myf-5, myogenin, and MRF-4. The forced expression of any one of these myogenic factors in a variety of nonmuscle cells results in myogenic conversion, as measured by the expression of skeletal muscle myosin. These experiments helped to establish the concept that the MyoD family members play a pivotal role in myogenesis (reviewed in Buckingham, 1992; Emerson, 1990; Olson and Klein, 1994; Weintraub, 1993; Weintraub *et al.*, 1991). The differential expression pattern of each of these proteins during embryonic development initially suggested nonoverlapping roles for each factor in the process of muscle formation (Buckingham, 1992). Subsequent analysis of

single and double knockout mutations in the mouse confirmed this idea and has clearly demonstrated that the MyoD family members play a critical role in both the determination and the differentiation of striated muscle cells during development (reviewed in Olson and Klein, 1994).

In the vertebrates, there is substantial evidence that MyoD family members function predominately as heterodimers with a set of ubiquitous bHLH proteins known as the E proteins (Lassar *et al.*, 1991). There are at present three known E-protein-encoding genes in vertebrates: E2A (differential splicing yields two isoforms, E12 and E47) (Murre *et al.*, 1989), E2-2 (Henthorn *et al.*, 1990), and HEB (Hu *et al.*, 1992). These gene products function in conjunction with cell type-specific factors, such as MyoD, in various developmental programs. In addition to these heterodimer functions, recent studies have also implicated E-protein homodimers in B-cell development (Choi *et al.*, 1996; Zhuang *et al.*, 1996, 1994).

MyoD and E-protein homologues have also been identified in invertebrates in which they are encoded by single-

¹ To whom correspondence should be addressed. Fax: (301) 402-3095. E-mail: bruce@sunspot.nci.nih.gov.

copy genes (Caudy *et al.*, 1988a, b; Cronmiller *et al.*, 1988; I. Araki *et al.*, 1994; Krause *et al.*, 1990, 1997; Michelson *et al.*, 1990; Paterson *et al.*, 1991; Venuti *et al.*, 1991). The most extensively studied invertebrate MyoD and E-protein homologues are those from *Drosophila* and *Caenorhabditis elegans*. In *Drosophila* the MyoD homologue, *nautilus* (*nau*), and the E-protein homologue, *daughterless* (*da*), are thought to heterodimerize to form a transcriptional activator, although no direct experimental evidence has confirmed this notion. Mutations in *da* are lethal and result in muscle defects (Abmayr *et al.*, 1995; Caudy *et al.*, 1988a).

The precise role of *nautilus* in *Drosophila* muscle development is currently under investigation: Deficiencies that cover *nau* do not block the specification or differentiation of the majority of somatic muscles; instead, only a subset of muscles fail to differentiate (Keller *et al.*, 1998).

Another approach using antisense and RNA interference studies in *Drosophila* indicates that *nautilus* is required for muscle formation in the embryo (Misquitta and Paterson, 1998).

In *C. elegans*, the MyoD homologue is CeMyoD, encoded by the *hlh-1* gene, and the E-protein homologue is CeE/DA, encoded by the *hlh-2* gene (Krause *et al.*, 1990, 1997). A null allele of *hlh-1*, *cc450*, has been identified and results in lethality associated with defects in myogenesis, although striated muscle cells are still formed (Chen *et al.*, 1994). No null mutations have been identified in *hlh-2*. Surprisingly, the embryonic expression pattern of *hlh-2* does not include differentiating striated muscle cells or their immediate precursors (Krause *et al.*, 1997).

We have explored the functional conservation and dimerization specificity of these myogenic factors using both *in vivo* and *in vitro* assays. DNA binding of homo- and heterodimer combinations of the MyoD and E proteins was assayed *in vitro*. Two *in vivo* assays were used: myogenic conversion of mouse 10T $\frac{1}{2}$ fibroblasts and rescue of the *C. elegans* *hlh-1(cc450)* null allele mutant. Using these approaches to directly compare factors from multiple species we demonstrate that different members of the MyoD and E-protein families can function in heterologous systems.

MATERIALS AND METHODS

Plasmids and Transfections

The isolation and sources for cDNAs were as follows: chicken MyoD (Lin *et al.*, 1989), chicken myogenin (Shirakata *et al.*, 1993), *Drosophila* Nautilus (Paterson *et al.*, 1991), *Drosophila* Daughterless (Caudy *et al.*, 1988a; Cronmiller *et al.*, 1988), *C. elegans* CeMyoD (Krause *et al.*, 1990), Id-1 (Benezra *et al.*, 1990), and *C. elegans* CeE/DA (Krause *et al.*, 1997).

Expression constructs for expression in 10T $\frac{1}{2}$ cells utilized the SV40 promoter, the RSV promoter, or the EMSV promoter (Lin *et al.*, 1989). 10T $\frac{1}{2}$ cells were grown in 60-mm dishes in DMEM with 10% fetal calf serum plus gentamycin and were transfected with 1–5 μ g of plasmid using either the calcium phosphate method (Lin *et al.*, 1989) or Lipofectamine as described by the manufacturer (Life Technologies). Cells were assayed for myosin expression using

the monoclonal antibody MF20 (Lin *et al.*, 1989); Nautilus was detected using the rabbit polyclonal antibody previously described (Paterson *et al.*, 1991).

Protein Expression and Analysis

Protein expression in *Escherichia coli* used the pRSET vectors (Invitrogen) in BL21(LysS) cells. Complete cDNAs for the various protein were cloned in frame into convenient sites of the pRSET multiple cloning site. Induction and protein isolation were as previously described (Shirakata *et al.*, 1993). Protein stocks were adjusted to 1 mg/ml with His-tag elution buffer for immediate use or dialyzed against 6 M urea, 20 mM Tris, pH 7.5, 150 mM NaCl followed by twofold dilutions with buffer containing no urea, to a final urea concentration of 0.75 M. Proteins stable under these conditions were dialyzed against 20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol and frozen at -80°C in small aliquots.

For baculovirus expression, (1) the cDNAs were cloned into the Pharmingen Vector 1392 modified with a six-His-tag oligo, and virus was plaque purified as described by the manufacturer, or (2) the Bac-to-Bac system (Life Technologies) was used as recommended by the manufacturer. Dephosphorylation of proteins was achieved by treatment with calf intestinal phosphatase as described (Mitsui *et al.*, 1993).

Myogenic Conversion Analysis

Four independent cultures were scored for myosin-positive cells and the average is presented in Table 1. Transfection efficiencies were very comparable ($\pm 10\%$) per plate as scored by GFP fluorescence (Clontech; pEGFP1) in companion cultures. Conversion rates were normalized to wild-type MyoD (CMD1), which was set to 100%.

RESULTS

Homo- and Heterodimer Requirements for Myogenic Conversion of Mouse 10T $\frac{1}{2}$ Fibroblasts

Expression of any of the vertebrate MyoD family members under the appropriate culture conditions has been shown to convert mouse 10T $\frac{1}{2}$ fibroblasts into myoblasts that can differentiate into myosin-positive, multinucleated cells (Emerson, 1990; Olson and Klein, 1994; Weintraub, 1993). Previous studies demonstrated that CeMyoD was also active in this assay, with a conversion frequency that was about 30-fold less than that obtained with mouse MyoD (Krause *et al.*, 1992), whereas *nautilus* was unable to convert this cell line (Shirakata and Paterson, 1995). It was proposed that the inability of *nautilus* to initiate myogenic conversion was due to inefficient dimerization with the endogenous vertebrate E factors due to key differences in the nonconserved residues in the bHLH domain of *nautilus* compared with vertebrate MyoDs (Shirakata *et al.*, 1993).

We have extended these earlier studies in order to determine the relative conversion frequency of vertebrate, *Drosophila*, and *C. elegans* myogenic factors and to investigate dimerization partner specificity. We have used the chicken MyoD factor (CMD1) as a representative vertebrate myo-

TABLE 1
Myogenic Conversion of 10T $\frac{1}{2}$ Cells

	No. of myosin-positive cells per dish (<i>n</i> = 4)	Relative conversion rate (%)
CMD1	459	100
CMD1 + Id-1	65	14
Nautilus	0	0
Nautilus + E12	4	0.9
Nautilus + Daughterless	43	9.4
Nautilus + Daughterless + Id-1	40	8.7
Nautilus + CeD/DA	78	17
CeMyoD	32	7
CeMyoD + Id-1	28	6.1
CeMyoD + E12	24	5.2
CeMyoD + Daughterless	5	1.1
CeMyoD + CeD/DA	36	7.8
Id-1	0	0
E12	0	0
Daughterless	0	0
CeE/DA	0	0
Vector (RSV or EMSV)	0	0

genic factor; both avian and mouse MyoD have similar conversion frequencies under our culture conditions (there is an overall 80% concordance between the amino acid sequences of MyoD and CMD1). Conversion is measured as the number of myosin-positive cells per 60-mm culture dish. The frequency of conversion of CMD1 is arbitrarily given a value of 100% (Table 1).

Transfection of 10T $\frac{1}{2}$ cells with *Drosophila nautilus* expression constructs resulted in no detectable myogenic conversion, as previously reported (Shirakata and Paterson, 1995), even though the protein was produced at high levels in the cell nuclei (data not shown). To test the hypothesis that *nautilus* requires *daughterless* as a dimerization partner, expression constructs for *nau* and *da* were transfected together into 10T $\frac{1}{2}$ cells. Although neither protein alone resulted in myogenic conversion, the two together resulted in almost 10% of the number of myosin-positive cells in vertebrate CMD1 (Table 1).

Other E proteins were also tested for their ability to augment *nau* conversion of 10T $\frac{1}{2}$ fibroblasts, including chicken E12 and *C. elegans* CeE/DA. When *nau* was cotransfected with a chicken E12 expression construct the frequency of conversion was about 1% compared to no conversion with *nau* alone (Table 1). This slight enhancement by E12 may reflect an ability of *nautilus* to weakly heterodimerize with chicken E12, when tested *in vitro* (see below). When *nau* was cotransfected with an expression construct for CeE/DA, the conversion frequency rose to 17%. As shown in Table 1, this was the most effective combination of invertebrate factors in the 10T $\frac{1}{2}$ myogenic conversion assay.

Although CeMyoD alone was able to convert 10T $\frac{1}{2}$ cells to muscle, it was possible that, like *nautilus*, conversion might be augmented by CeE/DA. Cotransfection of CeE/DA and CeMyoD expression constructs together had essentially no effect on the efficiency of 10T $\frac{1}{2}$ myogenic conversion (Table 1). Similarly, chicken E12 or *Drosophila daughterless* had no positive effect on the CeMyoD conversion frequency and the latter actually decreased conversion from (7% for CeMyoD alone to 1.1% for the combination of CeMyoD with *daughterless*.) Therefore, the ability of CeMyoD to convert 10T $\frac{1}{2}$ cells is not augmented by any E-protein family member tested, vertebrate or invertebrate.

These results support the interpretation that CeMyoD functions as a homodimer in 10T $\frac{1}{2}$ cell conversion, consistent with the absence of CeE/DA in myogenic cells in *C. elegans* (Krause *et al.*, 1997). To further test this idea we coexpressed Id-1, a known inhibitor of E-protein function in MyoD/E protein heterodimer formation (Benezra *et al.*, 1990), along with vertebrate CMD1, *nautilus* plus *daughterless*, or CeMyoD in the 10T $\frac{1}{2}$ conversion assay. Although Id-1 expression inhibited myogenic conversion by CMD1 by more than 80%, there was no affect on conversion by CeMyoD or *nautilus* plus *daughterless* (Table 1), supporting the interpretation that CeMyoD acts as a homodimer to activate myogenic conversion in 10T $\frac{1}{2}$ cells. Unexpectedly, based upon the *daughterless* requirement for *nautilus* myogenic conversion of 10T $\frac{1}{2}$ cells, Id-1 did not interfere with *daughterless* function in this assay. Preliminary studies indicate E12/E47 proteins have a higher affinity for Id-1 than for *da* (unpublished observations).

Interspecific Rescue of a *C. elegans* Mutant Lacking CeMyoD Activity

A mutation in the *C. elegans hlh-1* gene encoding CeMyoD was previously identified and shown to be due to a nonsense codon in the first exon (Chen *et al.*, 1994). There is no maternal contribution to CeMyoD function during embryogenesis (Chen *et al.*, 1994). Hence, this mutation (*cc450*) is a null allele by genetic and molecular criteria. Homozygous animals arrest and die after hatching at variable larval stages, most frequently as L1 larvae. These mutant animals display various morphogenic and elongation defects with short, fat bodies (a Dumpy phenotype) with random lumps and constrictions (Fig. 1A). In addition, muscle function is not normal, resulting in animals with severe movement defects. The few animals that do survive to adulthood are essentially immobile and have very small brood sizes. The *hlh-1(cc450)* homozygous animals can be fully rescued by a construct containing the genomic wild-type *hlh-1* gene or by cDNA expression directed by *hlh-1* promoter sequences (Chen *et al.*, 1994).

Because the *hlh-1(cc450)* mutant phenotypes are easily scored, the rescue of this mutant provides a sensitive assay for MyoD function. The four avian myogenic factors (MyoD, myogenin, Myf-5, and Mrf4) and *Drosophila nautilus* were cloned as cDNAs under the control of the *hlh-1*

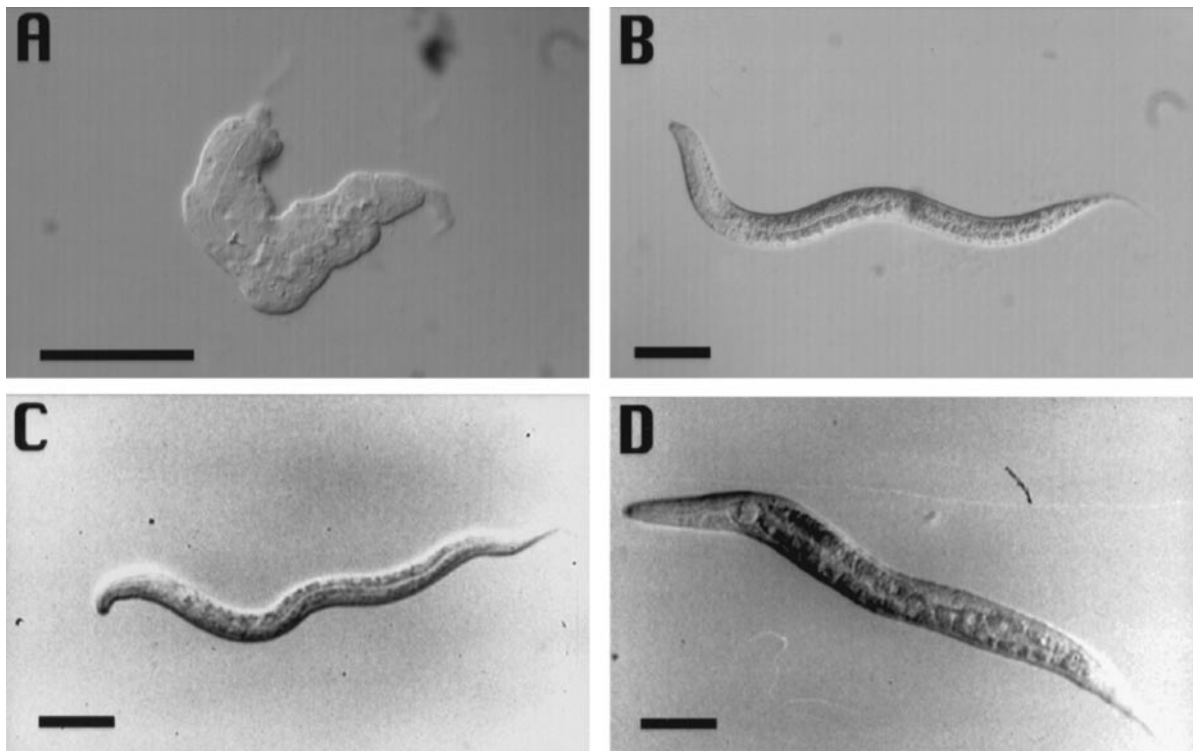


FIG. 1. Rescue of *C. elegans* *hlh-1(cc450)* null mutants with *nautilus* and chicken myogenin. (A) Homozygous *hlh-1(cc450)* mutant animal arrested after hatching as a short, fat, and partially paralyzed animal. (B) Wild-type L1 animal for comparison. (C) Transgenic animal expressing *Drosophila nautilus* under the control of the *hlh-1* promoter. Young larvae are often indistinguishable from wild-type animals. (D) Transgenic animals expressing chicken myogenin under the control of the *hlh-1* promoter. Although rescue of early larvae can be quite good, later stage larvae (shown) progressively show visible phenotypes with slight Dumpy-ness (short and fat) with poor movement and fertility.

promoter and tested for their ability to rescue the *hlh-1(cc450)* homozygous mutants. Coexpression of a construct encoding chicken E12 was also tested with each myogenic factor to see if it could enhance rescue. Two of the five tested myogenic factors, chicken myogenin and *Drosophila nautilus*, were able to rescue the lethality, movement, and fertility phenotypes of *hlh-1(cc450)* mutants (Table 2); the presence of chicken E12 had little to no effect for all myogenic factors.

Rescue with chicken myogenin was the weaker of the two factors, with movement and morphology defects partially restored, primarily in young larval animals. A few animals with the myogenin transgene survived to adulthood and fertility was increased compared with the mutant, but these adult animals still had pronounced movement, morphology, and fertility defects (Fig. 1D). The rescue with *Drosophila nautilus* was significantly better than with chicken myogenin. The early larval stages of *nautilus*-rescued animals were particularly well rescued and were often indistinguishable from wild-type controls (Fig. 1B versus 1C). At later stages, however, partial defects in movement, morphology, and fertility were apparent for the *nautilus*-rescued mutant (data not shown). We have not

further investigated the failure of the remaining chicken myogenic factors to rescue the *hlh-1(cc450)* mutation. It is possible that this failure to rescue reflects an inability of each of these factors to function in *C. elegans* myogenesis. Alternatively, this could reflect failure in transcription, translation, folding, posttranslational modification, or stability of the corresponding transgene products.

Dimerization Specificity and the Effects of Phosphorylation on MyoD and E-Family Member Interactions

We assessed dimerization specificity and DNA binding *in vitro* by measuring the ability of the various protein combinations produced in *E. coli* to shift an oligo containing the muscle creatine kinase enhancer right E-box (Lassar et al., 1991). CMD1 and *nautilus* each bound DNA poorly as homodimers; both readily formed DNA-binding heterodimers when provided an equal molar amount of the corresponding E protein (Fig. 2). Somewhat surprisingly, the species of origin of the E-protein partner for CMD1 and *nautilus* made no difference in binding efficiency by this assay; chicken E12, *Drosophila daughterless*, and *C. el-*

TABLE 2
Rescue of the *C. elegans hlh-1(cc450)* Mutant with Heterologous Myogenic Factors

	Rescue alone	Rescue with chicken
E12		
<i>C. elegans hlh-1</i>	+++++	++++
<i>Drosophila nautilus</i>	+++	+½
Chicken <i>myogenin</i>	+++	+/-
Chicken <i>MyoD</i>	-	-
Chicken <i>Myf-5</i>	-	-
Chicken <i>Mrf-4</i>	-	-
Mouse <i>MyoD</i>	-	-

Note. The degree of suppression is scored using the following scale: (+++++) complete rescue of lethality with wild-type appearance; (+++++) rescue with slight movement and body morphology defects; (++++) partial rescue with severe movement, body morphology, and fertility defects; (++) poor rescue with severe movement, body morphology, and fertility defects; (+/-) marginal rescue of lethality; (-) no rescue of lethality with animals indistinguishable from the original mutant phenotype. These are qualitative measurements scoring a combination of several phenotypes assayed over the course of development; a difference in score corresponding to one + is not considered significant.

egans CeE/DA each interacted equally well with the chicken or *Drosophila* myogenic factors, with the exception of *nautilus* with E12 (Fig. 2, lane 5).

In contrast to chicken MyoD and *Drosophila nautilus*, CeMyoD bound DNA avidly as a homodimer (Fig. 2, lane 9; also see Krause *et al.*, 1997). Complete conversion to heterodimers with an E partner could be achieved only when the ratio of E protein to CeMyoD was greater than 25:1; the CeE/DA:CeMyoD heterodimer shown used a ratio of 50:1 (Fig. 2, lane 12).

Phosphorylation of specific residues can affect dimerization properties of bHLH factors. CMD1 produced in Sf9 insect cells using the baculovirus system is phosphorylated on serines, qualitatively similar to the endogenous pattern; this phosphorylation allows the formation of efficient CMD1:E12 heterodimers while minimizing the formation of the CMD1 homodimer (Mitsui *et al.*, 1993). We have assayed dimerization preferences of phosphorylated and dephosphorylated forms of the myogenic factors that are the focus of this study. Extracts containing 10-fold more phosphorylated CMD1 than is shown in Fig. 2 (lane 1) for the bacterial protein do not promote homodimer formation yet allow heterodimerization with E protein to occur (Fig. 3A, lanes 1 and 3). Dephosphorylation of CMD1 by calf intestinal phosphatase results in the formation of a strong CMD1 homodimer band as well as an increase in heterodimer formation (Fig. 3A, lanes 2 and 4). *nautilus* produced in Sf9 cells behaves similarly to CMD1 and does not form homodimers efficiently *in vitro* unless it is dephosphorylated (Fig. 3B, lanes 1 and 3). Although the precise serines of CMD1 that mediate this effect have not been

identified, the phosphorylated domain responsible for this regulation is outside the conserved bHLH region (J.-M.Z. and B.M.P., in preparation), where there is only minimal amino acid sequence conservation between CMD1 and *nautilus*.

Like CMD1 and *nautilus*, CeMyoD homodimer formation is enhanced by dephosphorylation (Fig. 3C, lanes 1 and 2). However, the phosphorylated forms of CeMyoD produced in Sf9 cells do not interact with CeE/DA any differently from dephosphorylated or bacterially produced protein (Fig. 3C, lanes 3 and 4). That is, regardless of its phosphorylation status, CeMyoD preferentially forms homodimers and fails to heterodimerize efficiently with CeE/DA *in vitro*.

We have also investigated the effects of phosphorylation status on the dimerization specificity of chicken myogenin because it was able to partially rescue the *C. elegans hlh-1(cc450)* mutant. Unlike other avian myogenic factors, chicken myogenin is able to form homodimers regardless of its phosphorylation status or presence of an E protein (Fig. 3D). In this respect, chicken myogenin resembles CeMyoD. Unlike CeMyoD, however, chicken myogenin preferentially forms heterodimers with E12 when present in an equal molar concentration.

DISCUSSION

The ability to initiate myogenic conversion of the mouse fibroblast cell line 10T½ is a hallmark feature of the MyoD family of bHLH transcription factors. Using this conversion assay we have compared directly the function of vertebrate (avian), *Drosophila*, and *C. elegans* myogenic factors either alone or in combination with E-protein family members. We find that *C. elegans* CeMyoD alone, or *Drosophila nautilus* and *daughterless* together, can mediate myogenic conversion of this mouse cell line, with conversion frequencies of 7 and 10%, respectively, compared with vertebrate myogenic factors. The inability of *C. elegans* CeE/DA or any other E protein to augment the activity of CeMyoD is consistent with the notion that CeMyoD functions as a homodimer rather than a heterodimer (Krause *et al.*, 1997). This conclusion is reinforced by the observation that the E-protein inhibitor Id-1 does not affect myogenic conversion by CeMyoD, whereas the function of vertebrate MyoD is inhibited by more than 80% in this assay. Surprisingly, the *daughterless*-dependent conversion of 10T½ cells by *nautilus* is not affected by Id-1, likely reflecting the weak interaction of Id-1 with *daughterless* compared to the vertebrate E proteins (unpublished observations). It has been shown previously that *nautilus* lacks particular non-conserved hydrophilic residues in the bHLH domain that are critical in the vertebrate myogenic factors for dimerization specificity with vertebrate E12 (Shirakata and Pater-son, 1995; see below). Our results demonstrate that upon provision of an appropriate dimerization partner, in this case *daughterless* or *C. elegans* CeE/DA, *Drosophila nau-*

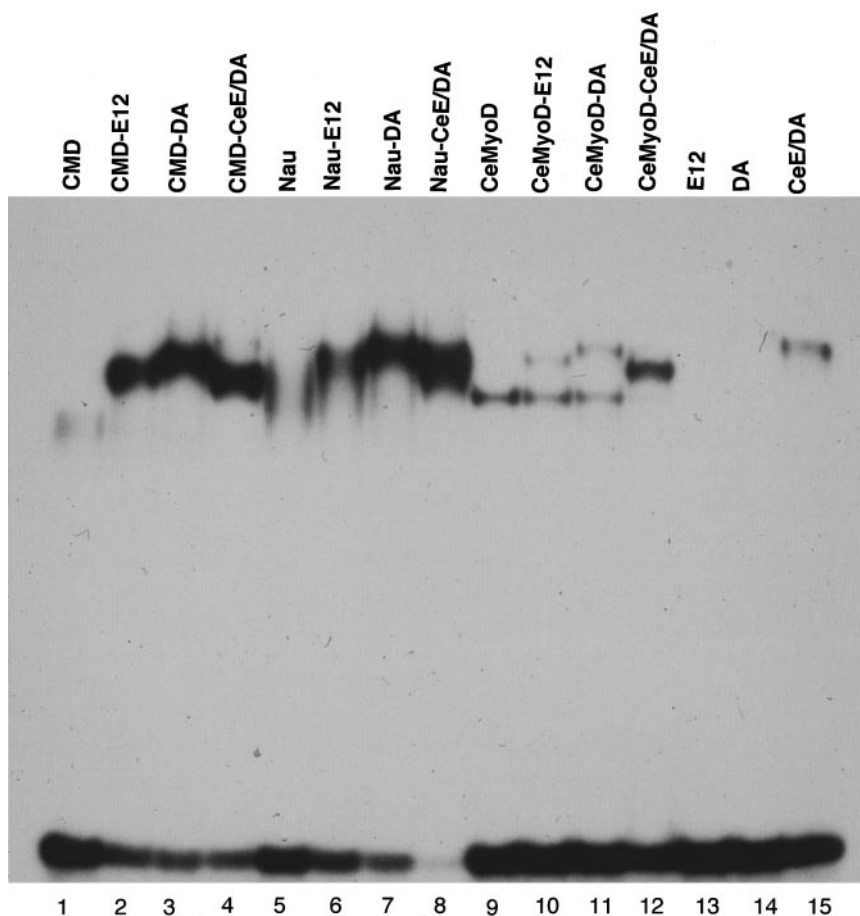


FIG. 2. DNA binding activity of the various myogenic factors with and without the designated E-protein partner. Factor combinations present in each lane are identified above each lane. Approximately 20 ng of each protein was used per binding reaction with the following exceptions: lane 1, 50 ng CMD1; lane 5, 200 ng *nautilus*; lane 10, 500 ng E12; lane 11, 500 ng *daughterless*; lane 12, 1 μ g CeE/DA. Note that efficient heterodimerization between CeE/DA and CeMyoD requires a ratio of 50:1 (lane 12). Increased levels of the single proteins were used to emphasize the lack of homodimer formation compared to concentrations used to form heterodimers. E12 has an inhibitory domain that minimizes homodimer formation and is also found in *daughterless* but is absent in CeE/DA (Shirakata and Paterson, 1995).

tilus is capable of efficient myogenic conversion of this mouse cell line.

As an additional test of function *in vivo*, we have assayed these myogenic factors for their ability to rescue a *C. elegans* mutant homozygous for a null allele of *hlh-1* encoding CeMyoD. Both *Drosophila nautilus* and chicken myogenin are able to partially rescue all of the mutant phenotypes. Rescue was noticeably better in younger larvae, which were often indistinguishable from wild-type animals. A limitation of this assay is that conclusions can be drawn only from positive results. The failure of the remaining three vertebrate myogenic factors (CMD1, Myf-5, and MRF-4), either alone or in combination with E-protein transgenes, to rescue the *C. elegans* mutant may simply reflect lower levels of expression from the transgenic arrays formed in *C. elegans*. We have not attempted to determine the expression level of the various heterologous

factors in transgenic animals because it would be difficult to interpret the results. For example, we are unsure if the heterologous factors, when expressed in *C. elegans*, are subject to proper posttranslational modifications necessary for function (e.g., phosphorylation). Therefore, detection of a particular factor does not ensure that it is present in a functional form. Furthermore, we are aware of *C. elegans* mutants in which CeMyoD protein is reduced to levels undetectable by our specific antibody yet is still sufficient for function and normal development (Harfe, Fire, and Krause, unpublished observation). Therefore the ability to detect, or not detect, a particular factor cannot necessarily be correlated with its ability to rescue. The results do, however, clearly demonstrate that both invertebrate and vertebrate myogenic factors are capable of functioning during *C. elegans* myogenesis.

Previous work on the vertebrate myogenic factors, and

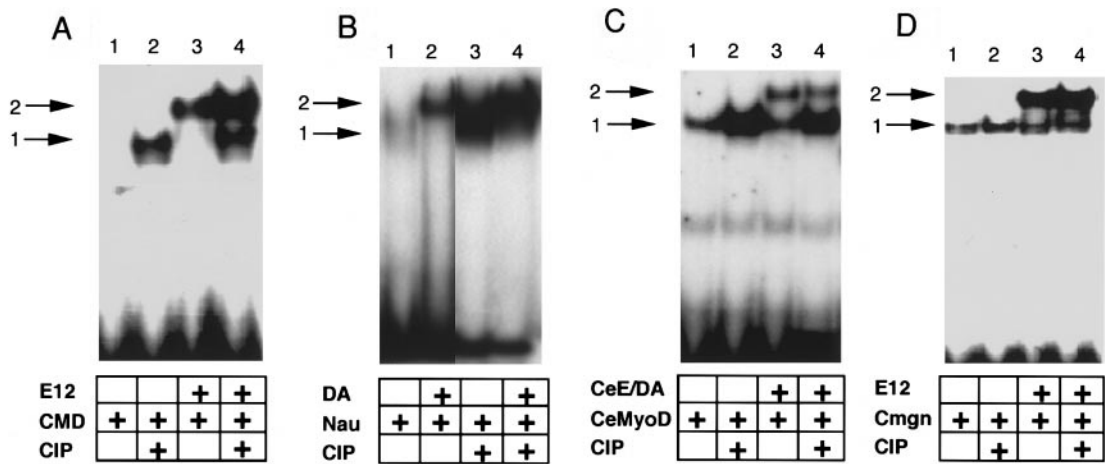


FIG. 3. The effects of phosphorylation on the homo- and heterodimerization of vertebrate, *Drosophila*, and *C. elegans* myogenic factors. The myogenic factors were produced in Sf9 insect cells, in which they are phosphorylated in a manner similar to that of proteins isolated from vertebrate muscle cells (Mitsui *et al.*, 1993). These proteins were tested for DNA binding before and after calf intestinal phosphatase (CIP) treatment, either alone or in combination with the relevant E protein produced in *E. coli*, as indicated above each blot. (A) Chicken MyoD (CMD1) and chicken E12: MyoD (CMD1) homodimers (arrow 1) formed efficiently only after CIP treatment. Note the enhanced formation of the heterodimer (arrow 2) after dephosphorylation of the MyoD (CMD1). (B) *Drosophila nautilus* and *daughterless*: similar to chicken MyoD, *nautilus* homodimer formation (arrow 1) is greatly enhanced upon dephosphorylation and there is a noticeable increase in heterodimer formation as well (arrow 2). (C) *C. elegans* CeMyoD and CeE/DA: each lane used a CeE/DA:CeMyoD ratio of 25:1 to force heterodimerization (arrow 2). Homodimers of CeMyoD (arrow 1) are favored under all conditions tested and are enhanced by dephosphorylation. (D) Chicken myogenin (Cmgn) and E12: Cmgn homodimers (arrow 1) are present at similar levels under all conditions tested although Cmgn:E heterodimers (arrow 2) are preferred when both factors are present in equimolar amounts.

our results here, strongly suggest that both *nautilus* and chicken myogenin function *in vivo* as obligate heterodimers with *daughterless* and E proteins, respectively. In contrast, the evidence presented here supports the previous suggestion that CeMyoD functions as a homodimeric myogenic factor (Krause *et al.*, 1997). Given these differences in E partner requirements it is surprising that chicken myogenin and *Drosophila nautilus* can rescue the *C. elegans hhl-1(cc450)* mutant. One possibility is that *nautilus* and chicken myogenin can efficiently homodimerize in the *C. elegans* environment. *nautilus* and CeMyoD share sequences and charge similarity at several residues in the bHLH domain that are known to be important for homo- versus heterodimer formation with E12 (Shirakata *et al.*, 1993). We demonstrate here that chicken myogenin shows phosphorylation-independent homodimerization (Fig. 3D). It may be a propensity for homodimerization by *nautilus* and chicken myogenin that allows these heterologous factors to efficiently substitute for CeMyoD function in *C. elegans*. Alternatively, the ability to rescue may be unrelated to dimerization status and instead reflect the ability of these two heterologous factors to best interact with other factors involved in promoting striated myogenesis in *C. elegans*.

Many factors are likely to interact with the myogenic bHLH proteins in the process of myogenesis, and several such factors, such as Mef-2 (Molkentin *et al.*, 1995) and

CBP/p300 (Eckner *et al.*, 1996; Puri *et al.*, 1997; Yuan *et al.*, 1996), have already been identified as important cofactors required for myogenesis. Given the high degree of evolutionary conservation of the bHLH domain and DNA binding properties among all myogenic factors, interspecific function is not unexpected in simple *in vivo* assays such as transactivation of a target gene promoter. However, in light of the many factors that must come together to drive myogenesis, it is surprising that myogenic factors sharing little apparent homology outside of the bHLH domain (e.g., chicken myogenin, *Drosophila nautilus*, and *C. elegans* CeMyoD) can substitute for one another within the context of the developing organism. This suggests that either the necessary interactions among the various transcription factors and basal machinery that regulate myogenesis are highly conserved or that the myogenic factors alone, and specifically the bHLH domains, are the major effectors of myogenesis.

REFERENCES

Abmayr, S. M., Erickson, M. S., and Bour, B. A. (1995). Embryonic development of the larval body wall musculature of *Drosophila melanogaster*. *Trends Genet.* **11**, 153–159.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49–59.

- Buckingham, M. (1992). Making muscle in mammals. *Trends Genet.* **8**, 144–148.
- Caudy, M., Grell, E. H., Dambly-Chaudiere, C., Ghysen, A., Jan, L. Y., and Jan, Y. N. (1988a). The maternal sex determination gene *daughterless* has zygotic activity necessary for the formation of peripheral neurons in *Drosophila*. *Genes Dev.* **2**, 843–852.
- Caudy, M., Vassin, H., Brand, M., Tuma, R., Jan, L. Y., and Jan, Y. N. (1988b). *daughterless*, a *Drosophila* gene essential for both neurogenesis and sex determination, has sequence similarities to *myc* and the *achaete-scute* complex. *Cell* **55**, 1061–1067.
- Chen, L., Krause, M., Draper, B., Weintraub, H., and Fire, A. (1992). Body-wall muscle formation in *Caenorhabditis elegans* embryos that lack the MyoD homolog *hlh-1*. *Science* **256**, 240–243.
- Chen, L., Krause, M., Sepanski, M., and Fire, A. (1994). The *Caenorhabditis elegans* MYOD homologue HLH-1 is essential for proper muscle function and complete morphogenesis. *Development* **120**, 1631–1641.
- Choi, J. K., Shen, C. P., Radomska, H. S., Eckhardt, L. A., and Kadesch, T. (1996). E47 activates the Ig-heavy chain and TdT loci in non-B cells. *EMBO J.* **15**, 5014–5021.
- Cronmiller, C., Schedl, P., and Cline, T. W. (1988). Molecular characterization of *daughterless*, a *Drosophila* sex determination gene with multiple roles in development. *Genes Dev.* **2**, 1666–1676.
- Eckner, R., Yao, T. P., Oldread, E., and Livingston, D. M. (1996). Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. *Genes Dev.* **10**, 2478–2490.
- Emerson, C. P. (1990). Myogenesis and developmental control genes. *Curr. Opin. Cell Biol.* **2**, 1065–1075.
- Henthorn, P., Kiledjian, M., and Kadesch, T. (1990). Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science* **247**, 467–470.
- Hu, J. S., Olson, E. N., and Kingston, R. E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. *Mol. Cell. Biol.* **12**, 1031–1042.
- I. Araki, H. S., Makabe, K. W., and Satoh, N. (1994). Expression of AMD1, a gene for a MyoD1-related factor in the ascidian *Halicynthia roretzi*. *Roux's Arch. Dev. Biol.* **203**, 320–327.
- Keller, C. A., Grill, M. A., and Abmayr, S. M. (1998). A role for *nautilus* in the differentiation of muscle precursors. *Dev. Biol.* **181**, 181.
- Krause, M., Fire, A., Harrison, S. W., Priess, J., and Weintraub, H. (1990). CeMyoD accumulation defines the body wall muscle cell fate during *C. elegans* embryogenesis. *Cell* **63**, 907–919.
- Krause, M., Fire, A., White-Harrison, S., Weintraub, H., and Tapscott, S. (1992). Functional conservation of nematode and vertebrate myogenic regulatory factors. *J. Cell Sci. Suppl.* **16**, 111–115.
- Krause, M., Park, M., Zhang, J. M., Yuan, J., Harfe, B., Xu, S. Q., Greenwald, I., Cole, M., Paterson, B., and Fire, A. (1997). A *C. elegans* E/Daughterless bHLH protein marks neuronal but not striated muscle development. *Development* **124**, 2179–2189.
- Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**, 305–315.
- Lin, Z. Y., Dechesne, C. A., Eldridge, J., and Paterson, B. M. (1989). An avian muscle factor related to MyoD1 activates muscle-specific promoters in nonmuscle cells of different germ-layer origin and in BrdU-treated myoblasts. *Genes Dev.* **3**, 986–996.
- Michelson, A. M., Abmayr, S. M., Bate, M., Arias, A. M., and Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes Dev.* **4**, 2086–2097.
- Misquitta, L., and Paterson, B. M. (1999). Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-1): A role for *nautilus* in embryonic somatic muscle formation. *Proc. Natl. Acad. Sci. USA* **96**, 1451–1456.
- Mitsui, K., Shirakata, M., and Paterson, B. M. (1993). Phosphorylation inhibits the DNA-binding activity of MyoD homodimers but not MyoD-E12 heterodimers. *J. Biol. Chem.* **268**, 24415–24420.
- Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**, 1125–1136.
- Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., et al. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537–544.
- Olson, E. N., and Klein, W. H. (1994). bHLH factors in muscle development: Dead lines and commitments, what to leave in and what to leave out. *Genes Dev.* **8**, 1–8.
- Paterson, B. M., Walldorf, U., Eldridge, J., Dubendorfer, A., Frasch, M., and Gehring, W. J. (1991). The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Natl. Acad. Sci. USA* **88**, 3782–3786.
- Puri, P. L., Avantiaggiati, M. L., Balsano, C., Sang, N., Graessmann, A., Giordano, A., and Leverro, M. (1997). p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. *EMBO J.* **16**, 369–383.
- Shirakata, M., Friedman, F. K., Wei, Q., and Paterson, B. M. (1993). Dimerization specificity of myogenic helix-loop-helix DNA-binding factors directed by nonconserved hydrophilic residues. *Genes Dev.* **7**, 2456–2470.
- Shirakata, M., and Paterson, B. M. (1995). The E12 inhibitory domain prevents homodimer formation and facilitates selective heterodimerization with the MyoD family of gene regulatory factors. *EMBO J.* **14**, 1766–1772.
- Venuti, J. M., Goldberg, L., Chakraborty, T., Olson, E. N., and Klein, W. H. (1991). A myogenic factor from sea urchin embryos capable of programming muscle differentiation in mammalian cells. *Proc. Natl. Acad. Sci. USA* **88**, 6219–6223.
- Weintraub, H. (1993). The MyoD family and myogenesis: Redundancy, networks, and thresholds. *Cell* **75**, 1241–1244.
- Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., et al. (1991). The myoD gene family: Nodal point during specification of the muscle cell lineage. *Science* **251**, 761–766.
- Yuan, W., Condorelli, G., Caruso, M., Felsani, A., and Giordano, A. (1996). Human p300 protein is a coactivator for the transcription factor MyoD. *J. Biol. Chem.* **271**, 9009–9013.
- Zhuang, Y., Cheng, P., and Weintraub, H. (1996). B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. *Mol. Cell. Biol.* **16**, 2898–2905.
- Zhuang, Y., Soriano, P., and Weintraub, H. (1994). The helix-loop-helix gene E2A is required for B cell formation. *Cell* **79**, 875–884.

Received for publication August 11, 1998

Revised January 7, 1999

Accepted January 22, 1999